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A Morphological and Histological Study of the Hepatitis B Antigen Treated Nicotiana sylvestris and Nicotiana tabaccum Variety Xanthi

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A MORPHOLOGICAL AND HISTOLOGICAL STUDY OF THE HEPATITIS B ANTIGEN TREATED *Nicotiana sylvestris* AND *Nicotiana tabaccum* VARIETY XANTHI

by

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VITA

The author, Jeanne Elisabeth Moldenhauer, is the daughter of Richard and Dorothy Gibbons. She was born July 22, 1951, in Chicago, Illinois.

Her elementary education was obtained at Jahn School, and her secondary education at Carl Schurz High School where she graduated in June, 1969, with special honors in the science, mathematics and Latin city competition. Both are Chicago Public Schools.

She attended Trinity College in Deerfield, Illinois from September, 1969 until June, 1971. She then attended Wright College, Chicago, Illinois and received an Associate of Arts degree in June, 1972 with a double major of biology and mathematics. From September, 1972 until May, 1974 she attended the University of Missouri at St. Louis where she received a Bachelor of Arts degree with honors. She majored in biology and worked on a research project which resulted in the writing of a genetics lab manual with Dr. C. Granger.

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CHAPTER I

INTRODUCTION

Viruses are obligate parasites. They multiply only in living hosts. They are highly specific and can live only in certain hosts. Accordingly, there are insect, animal, plant and bacterial viruses, depending on the type of host in which they replicate.

There is no conclusive evidence for viruses of plants being able to replicate in animals, nor for animal viruses being able to replicate in plants. In fact a plant virus like tobacco mosaic virus causes a systemic disease in tobacco plants, a localized necrosis in pinto beans and Nicotiana glutinosa and has no effect on some other plants suggesting the specificity of viruses. Likewise, animal viruses are known for their specificity. Certain strains of influenza virus infect horses while other strains infect man. An animal virus, hepatitis B (HBV) is so specific that man is the only confirmed natural host.

In 1973, Banatvala and Payne reported that hepatitis B virus could be transmitted to tobacco plants and cause necrosis of the leaves. This was exciting, as it suggested that viruses could cross kingdom lines in the type of host which they infect. Many workers were unsuccessful in trying to repeat their experiments; Beasley and Su (1973), Howard

and McCarthy (1974) and Dhaliwal, Rubinstein and Dietz (1974). However, Kelkar et al. (1975) in India, was able to induce leaf necrosis in one species of tobacco plant, but was unsuccessful with a second species.

The purpose of this research is to confirm the replication of HBAg in Nicotiana sylvestris and Nicotiana tabaccum variety xanthi by studying the morphological, cytological and histochemical changes occurring in inoculated plants.

CHAPTER II

REVIEW OF THE RELATED LITERATURE

The under-mentioned literature pertains only to my research on the interaction of hepatitis B antigen with plants.

THE HEPATITIS ANTIGENS

Hepatitis may be caused by one of the hepatitis viruses (type A or B), or some other viruses such as yellow fever virus, cyclomegalo virus or Epstein-Barr virus (Lennette, 1974). In 1946, MacCullum and Miles reported that the virus-like agent (presumably hepatitis causing virus) could be transmitted only nine times in cell culture. Mosley (1967) speculated that this may be explained in terms of latent hepatitis, which requires an activation of the virus in rats by some unknown host component. In an attempt to understand hepatitis and possibly even control it, scientists (Colbert (1948): Maynard et al. (1975)) attempted to cultivate the human hepatitis agents in laboratory animals. However, the results obtained by these investigators were not confirmed.

Ward and Krugman (1962) reported that hepatitis type A could be transmitted parenterally although it is not the natural route. Blumberg et al. (1971) isolated an "Australia

Antigen" and he was able to show hepatitis type A and type B were caused by distinct agents. Okochi et al. (1970) found that there was a strong association between Australia antigen in the serum and post transfusion hepatitis.

Through electron microscope studies Bayer et al. (1968) found that Australia antigen contained 19 - 21 millimicrons diameter particles. They were round to ovoid in shape and some had "knob-like structures projecting 30 millimicrons". Almeida et al. (1969) saw antigen and tubular forms which were several thousand angstroms in length.

Krugman and Giles in 1970 reported the oral transmission of serum hepatitis.

Dane et al. (1970) discovered double-shelled virus-like particles (42 nm.) in the multiple serum specimens of patients with Australia-Antigen-Associated hepatitis. They postulated that these might be the complete virus particles while the 22 nm. particles and the long antigen particles were surplus.

VACCINATION CONTAINING HBAg: SEROLOGY

Krugman et al. (1971) prepared "active" vaccine from hepatitis type B strain MS-2 by boiling to inactivate the virus. Children were given two inoculations at four month intervals and they reported that hepatitis B was prevented in thirty-nine (out of forty-five) children in this way. Differential diagnostic procedures for type A and type B

hepatitis viruses were studied by Giles and Krugman (1972). They found type B had a longer incubation period than type A. While trying to correlate post-transfusion hepatitis with the Australia antigen, Gocke (1972) investigated the case histories of patients receiving transfusion of blood positive for Australia antigen and found that 52% of the patients developed hepatitis and 23% developed an immune response.

The aetiology of the human viral hepatitis was investigated by a committee chaired by Evans (1972). The Milan antigen that was cultivatable in marmosets, appeared to be an abnormal serum-lipoprotein and was associated with liver damage. However, it was found that this antigen was not specifically related to infectious hepatitis. Both type A and type B hepatitis produce pathological liver lesions, but they were not distinguishable. Clinical differentiation alone was usually not conclusive, as hepatitis was quite variable (Lennette, 1974).

Hirschman (1974) postulated that the DNA polymerase associated with HBAg might be used as a chemical tag for HBV. Thus, attention was focused on the Dane particle core which seemed to be enveloped by protein strands. In further studies, Hirschman et al. (1974) isolated infected liver cells and studied purified intranuclear particles. Ultraviolet absorption spectra showed peaks at 264 and 280 nm. The particles observed were 27 - 28 nm. in diameter.

Electron microscope studies on sera treated with chloroform and protease suggested that there was an antigen associated with the Dane particle coat but not associated with any of the small spheres or tubules (Moodie et al. (1974). They also noted it was destroyed by the protease, postulating it might account for the frequency of $HBAg^+$ sera with complexes of Dane particles only.

STABILITY OF HEPATITIS B ANTIGEN

Favero et al. (1974) performed several experiments to determine whether cleaning and disinfecting techniques used in the laboratory were inactivating the HBAg. The surfaces tested were stainless steel and cotton swabs. Exposure to constant conditions of 25°C and 42% R.H. only reduced the antigenic activity 15 to 20% and almost no antigenic activity was lost when the swabs were shipped for seven days. They were able to recover about 10% of the antigen after normal swab rinse techniques. Similarly, Krugman et al. (1970) found differences in heat resistance of type A and type B hepatitis. In a relatively small period of time, the virus was inactivated at 98°C. He found that type A was resistant when exposed to 56°C for 1 hour and type B withstood 60°C for 4 hours.

Cameron and Dane (1974) demonstrated the technique of radioimmunoassay for the measurement and detection of viral antigens and antibodies. They found it particularly useful

for HBAg detection.

INTERNATIONAL HEPATITIS SEMINARS

The year 1975 was important in hepatitis research. Two major worldwide seminars were held. Purcell (1975a) discussed the recent advances in hepatitis A research. He discussed Reinhardt's transmission of type A to marmosets and further stated that marmoset and human antigens were not identical. Similarly, Purcell (1975b) discussed the current understanding of hepatitis type B, correlating exposure and infection. Infection is associated with production of relatively large quantities of antigen. Prophylaxis is being studied using this antigen and surface antigens in chimpanzees and rhesus monkeys, hoping to prevent hepatitis as a current disease in man. Immunization studies by Purcell and Gerin (1975) presented a three-step procedure to remove (inactivate) the HBV in the starting human material: 1. Dane particles were removed by centrifugation, 2. The remaining non-infectious particles were then purified by centrifugation and as a final precaution, they were formalin treated. Potency of the prototype subunit vaccine was tested in guinea pigs. They found that the hepatitis B surface antigen, though inactivated, was capable of eliciting an immune response, 3. Tests of the vaccine's safety were performed using serologically-negative chimpanzees. The immunization was given in two doses, one month apart. No signs of infec-

tion were observed, however, the affected animals did develop hepatitis B surface antibody.

IS HEPATITIS REALLY A VIRAL DISEASE?

Portocalo (1974) reported some interesting theories based on his study of hepatitis. One premise is that hepatitis has liver involvement where hepatocytes have lesions of various intensities. Sometimes, large zones of cellular lysis are evidenced. This severity can be expressed by changes in serum protein concentrations (implying that structural hepatocyte changes cause functional changes and this change allows release of products into the blood). His investigation suggested that the physical and chemical properties of the Australia antigen makes it behave like a high molecular weight protein instead of a viral particle. The hypothesis was tested by studying the interaction of Australia-antigen and human serum, normal human serum and rabbit anti-human liver serum subjected to electroimmunodiffusion. Interaction of the Australia antigen with human liver antibodies supports the idea that a protein released by the liver causes the lesion rather than the hepatitis B virus.

CHARACTERIZATION OF THE CORE AND SURFACE ANTIGEN

Almeida and Waterson (1975) suggested that further research should be undertaken to study the possibility of perfecting HBsAg as a means of protection from hepatitis.

HBsAg is reported to consist of a minimum of nine polypeptides (two of which are thought to be glycoproteins), most of which contained the group specific 'a' and subtype specific 'd' or 'y' determinants inherent in their structure (Gerin et al. 1975). Purified preparations of HBsAg allowed Dreesman et al. (1975) to identify four to six different polypeptides, three glycoproteins, cholesterol, three polar lipids and two glycolipids composing two distinct antigen subtypes.

Liver tissue, obtained by autopsy and needle biopsy, from thirty-eight patients with HBV were studied by Huang (1975a) and he consistently found most of the HBcAg (21 - 25 nm.) in the liver cell nuclei and just a small amount in the cytoplasm. He found the HBsAg in the endoplasmic reticulum and used this for diagnosis and staining of the HBcAg was facilitated by protease treatment.

Several researchers studied the association between DNA polymerase and the core antigens (Hirschman et al., 1975; Robinson, 1975; and Schuurs and Wolters, 1975). Although the data was inconclusive, it was suggested that the DNA polymerase associated with HBAg was specific for its own DNA as a template.

INTERNATIONAL AGREEMENT ON NOMENCLATURE

The United States Communicable Disease Center

(MMWR, 1977) accepted the terminology agreed upon at an international convention. With respect to hepatitis B virus, the following nomenclature was accepted:

HBV:	Hepatitis B virus (42 nm. double shell, originally the Dane particle).
HBsAg:	The hepatitis B antigen found on the surface of the virus and on unattached 22 nm. spheres and tubules.
HBcAg:	Antigen within the core of the virus.
HBeAg:	'e' antigen closely associated with hepatitis B infection.
Anti-HBs:	Antibody to HBsAg.
Anti-HBc:	Antibody to HBcAg.
Anti-HBe:	Antibody to HBeAg.

DISEASE SYMPTOMOLOGIES

There are several objective signs leading to a clinical diagnosis of hepatitis. Seide (1967) reported that the clinical manifestations include fever, mucotaneous manifestations, cardiocirculatory disturbances, abdominal signs, urinary dysfunction, central nervous system manifestations (fatigue, apathy and depression), hematological changes and abnormalities of serum constituents like enzymes. Rossi (1972) reported that four proteins were affected: albumin decreased in concentration and alpha, beta, and gamma globulins increased in concentration. The enzymatic changes

noted were an increase in serum glutamic pyruvic transaminase, serum glutamic oxalacetic transaminase, lactic dehydrogenase, fructose-1-phosphate-aldolase, sorbital dehydrogenase, leucine aminopeptidase, ornithine dehydrogenase and a decrease in cholinesterase. The onset may be sudden or gradual with anorexia, headache, irritability, vomiting and abdominal discomfort.

Krugman and his associates (1960, 1961, 1962, 1967) performed several studies on hepatitis, providing evidence for two types of infectious hepatitis, MS-1 and MS-2 viruses, having different clinical, biochemical and immunological characteristics. The MS-1 virus had an incubation time of 31 to 38 days while MS-2 had an incubation period of 41 to 69 days and both were orally transmitted. The two were distinguished on the basis of serum transaminase activity and thymol turbidity. The patients which had been infected with MS-1 virus were immune to reinfection, while those with MS-2 were susceptible to MS-1.

HEPATITIS AND RELATED DISEASES

Four out of eleven patients with polyarteritis nodosa were also found to have Australia antigenemia (Gocke et al. 1970). The four who were positive for Australia antigen had typical polyarteritis syndrome but they differed from the others in that they had evidence of mild liver damage. In a second study (Gocke et al. 1971) on the association

between infectious agents and connective tissue disorders (vascularitis) they could not ascertain the mechanisms responsible. In a similar type of study, Waterson (1972) found that the susceptibility of the patient to hepatitis increases when the patient has New Castle's disease.

HEPATITIS DETECTION METHODS

Indirect methods measure changes in the system due to the viral infection. Krugman et al. (1967) studied the changes in serum glutamic oxalacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) levels. Both levels increase greatly but the SGPT is more specific for hepatocyte injury because, unlike the SGOT, it is not elevated in patients with cardiac damage. The ratio of SGOT to SGPT was discussed by DiRitis et al. (1957).

A white blood cell count also provides an indirect method of determining presence of the virus. In the pre-icteric phase, there is a characteristic leukopenia while towards the end of the phase, large atypical lymphocytes are present, similar to those with infectious mononucleosis. Serum bilirubin may be measured as it rises upon viral infection and bile can be detected in the urine before the concentration of serum bilirubin rises (Bodansky, et al. (1959).

Direct methods for the detection of hepatitis virus are also available. Counterelectrophoresis (Hyland Labora-

tories, 1973) detects hepatitis associated Australia antigen. It does not differentiate between types of hepatitis viruses. The principle involved in this method is that in a negatively charged gel, gamma globulins (antibodies) are displaced in movement toward the cathode by the endosmatic flow of buffer in the gel.

Rheophoresis is a specialized method of agar gel diffusion for the detection of hepatitis associated antigen (Jambazian and Holper, 1971) where the agar diffusion cup is surrounded by a circular moat filled with buffer and the entire surface is covered by a piece of plastic containing a hole directly over the antibody well. Dehydration of the antibody well caused production of hydrodynamic forces and concentration of the proteins toward the center of the plate. Sensitivity of this test was increased by Ashcavai and Peters (1973) by modifying the circular pattern into a hexagonal test pattern which allows higher concentrations of test serum to be delivered to the antibody in contrast to the usual immunodiffusion, with the serum being dissipated in all directions. Positive reactions are shown by precipitin rings.

The ability of complement to combine with antigen-antibody complexes can be used to detect immune reactions even when agglutination or precipitation tests are absent. An advantage of this test is that either soluble or insoluble antigens may be used. It depends upon two properties

of complement; the lysis of sensitized erythrocytes and the binding (fixation) of complement by antigen-antibody complexes. The test is quite sensitive and can even be used to determine the titre (Kabat and Mayers, 1967). Vyas and Shulman (1970) describe hemagglutination assays using red cells coated with isolated antigen-coupled with chromic chloride for antibody detection, while antigen detection is dependent upon the inhibition of hemagglutination. Cayzer et al. (1974) improvised on this technique using turkey erythrocytes coated with affinity-column-purified antibody to HBAg. Blood screening programs use this method rather than radioimmunoassays due to its speed, simplicity and cost, even though it is less sensitive.

RIA can be used in two ways (Purcell, et al. 1973 and 1974). One type is used to detect the Dane particle core antigen and the specific antibody. The core was found in Dane particle concentrates but not in preparations of the 22 nm. particles.

PATHOLOGICAL CHANGES

Pathologists studying hepatitis patients have found that the main changes occur in the liver. According to Edgington and Ritt (1971) agents such as the SH virus possess unique pathogenic potential beyond that of direct viral-induced tissue injury (demonstrated by snap frozen needle biopsies). The acute phase characteristically shows a great

swelling or ballooning of liver cells (Smetana, 1957). The cytoplasm has a ground glass appearance with lysis of the nuclei and cytoplasmic membranes, eosinophillic necrosis and foci of collapse which quite often are associated with infiltration of mononuclear cells. By the time jaundice appears, widespread parenchymal destruction may be evidenced. In most cases, a complete regeneration of liver occurs in two to three months. The regenerative changes are characterized by amitotic and mitotic activity with multinucleated cells appearing two to three weeks after the onset of jaundice.

Mallory (1947) describes subacute hepatitis as hepatitis resulting in death in three to eight weeks. The liver size is either reduced or abnormally large. Often it is very deformed, with yellow-green nodules surrounding red-depressed areas. Cut surfaces may appear granular with irregular parenchymal destruction. After death, the following changes may be seen; inflammation, stomach hemmorhages, epigastrointestinal wall hemorrhages, and lesions may be detected in the duodenum, jejunem and kidney. The most devastating type of hepatitis is fulminating hepatitis. Death occurs within ten days, liver size is reduced, acute yellow atrophy can be seen, cells are completely destroyed with no evidence of regeneration and numerous macrophages and erythrocytes are seen in lobular remnants (Locke and Mallory, 1946). Liver sections may be stained with eosin

and hematoxylin and studied under a microscope with 330X magnification, for cobblestone cytoplasmic patterns, enlarged nuclei, focal points and finely divided nuclear chromatin.

Three basic types of stains can be used for the detection of Australia Antigen: conventional paraffin sections, immunofluorescent microscopy or immunoelectron microscopy.

Shikata et al. (1974) compared several light microscope staining techniques on conventional formalin fixed paraffin embedded liver sections to detect cytoplasmic inclusion bodies (characteristic of infection by the Australia antigen). The dye's affinity for Australia antigen was confirmed by the fluorescent antibody technique. They found that the modified orcein method, Gomori's aldehyde-fuchsin method and the aldehyde-thionine method were the best. The stains were able to clearly demonstrate cytoplasmic inclusion bodies (thought to be Australian antigen aggregates) even though this is contrary to previous thoughts.

Huang (1975b) developed an immunohistochemical staining technique to demonstrate the hepatitis B core and surface antigens. This stain gave evidence to indicate that the HBsAg is produced exclusively in the cytoplasm of the liver cells. Ground glass cytoplasmic changes were shown with conventional hematoxylin eosin stains and

controls were done by staining some sections using Shikata et als. (1974) modified orcein method.

THE CULTIVATION OF HEPATITIS VIRUSES

Jenson (1970) grew organ cultures (fragments) of human inguinal lymph nodes, taken from four patients under five years of age, by placing them on human fibrin and foam which was maintained in a nutrient media for up to eighteen days. Electron microscope studies showed that the cells were viable. At this time, they were inoculated with either Herpes simplex or Australia antigen. The cultures were checked at 48 and 96 hours for the presence of virus. At both time intervals, all the cultures inoculated with Herpes simplex showed the presence of Herpes simplex and one of four cultures showed the presence of Australia antigen. They concluded that some lymph node organ cultures may support the production of Australia antigen but this was not confirmed. T-lymphocytes were stimulated with phytohemagglutinin in carriers of Australia antigen by Clot et al. (1973). They suggested that the HAA sera could inhibit the proliferation of normal lymphocytes.

Rubenstein et al. (1974) used rat and monkey livers to obtain liver cells for tissue cultures. The workers then tried to cultivate some viruses in the cells. They found that rat cells supported the growth of Sendai virus and monkey cells could maintain yellow fever virus. When

organ cultures (human embryo liver) were used, adenovirus type 5 and Herpes simplex could be maintained. Australia antigen failed to grow in cultures of liver cells from monkeys or humans.

Schable et al. (1974) used transformed chimpanzee lymphocytes to ascertain whether or not they would support hepatitis B virus replication. They continued their experiment for five months and could not detect surface antigens or antibodies of hepatitis. Deinhardt et al. (1975) used marmosets, but found that only some were susceptible to hepatitis A (given orally). Antigen presence was confirmed in fecal specimens. They used several different strains for inoculation. The recoverable antigen strains of MS-1 and CR-326 were antigenically similar or identical but the GB strain was quite different. Upon repeated infection, liver necrosis occurred in some animals while others became immune. Serial transmission was evidenced and the entire experiment was repeated by several laboratories (Purcell, 1975b).

The infectivity of non-human primates with virus-like particles associated with MS-1 hepatitis, was reviewed by Maynard et al. (1975). Both marmosets and chimpanzees were inoculated and upon isolation, 27 nm. particles of virus were observed under immunoelectronmicroscopy of liver and stool samples. They concluded that infectivity of these animals was a possibility.

Almeida and Waterson (1975) stated that HBAg cannot

be grown in vitro so workers must depend on serological, biochemical and electron microscopy. Conversely, Barker et al. (1975) observed the natural infection of chimpanzees with HBV and it seemed that this species might provide a sensitive model system for type B hepatitis. In most cases, chimpanzees which were negative for HBV, when inoculated, produced serological evidence of infection. Infection was evidenced by the appearance of circulating HBsAg and later antibodies (surface and core) were present in the serum. Thirty-one of forty-six inoculated animals showed enzyme changes characteristic of liver damage. The titre of HBV (both adw and ayw subtypes) used was $10^{7.5}$ infectious units per milliliter. They also tested Rhesus monkeys for infectivity of HBV but found them to be less sensitive than the chimpanzees.

Zuckerman (1973) studied HBV in both tissue and organ cultures. He cultivated human and non-human-primate liver cells. He demonstrated the presence of fluorescent anti HBcAg and fluorescent anti HBsAg attachment to the cytoplasm and nucleus. Some HBsAg was produced although not often in organ cultures. Serial passage could not be achieved in these cases. Serial passage has been achieved in some cases using pieces of human-liver-embryo (cultivated on CAM developing chick embryos).

Wicks et al. (1975) assessed thymus derived (T) lymphocyte function in healthy carriers of HBsAg in com-

parison to those with acute viral hepatitis B. The function was assessed using phytohemagglutinin (PHA) to induce blast cell transformation and also sheep cell rosette formation. The over-all cell mediated immune function (T-lymphocytes) was similar for three in vitro studies except for a decrease in the total number of T-cells in patients with acute hepatitis. They felt that this implied the virus was responsible for reduced production of T-cells.

In mid-1973, Banatvala and Payne reported that they had inoculated the tobacco plant, Nicotiana sylvestris with sera positive for hepatitis B antigen and the inoculated plants developed lesions. This was exciting news. It had been previously thought that the host specificity of viruses restricted them to one kingdom. Many other researchers tried to reproduce their experiments. Beasley and Su (1973) inoculated 150 plants (N. sylvestris) with 0.1 ml. of $HBAg^+$ sera. The environmental conditions used were 25°C, RH 85%, for 7 weeks after germination. They were subjected to a ten hour dark phase and were then inoculated. Their results showed no lesions. They postulated that the failure to get lesions was because they used subtype ad (a Taiwan strain) but Banatvala and Payne did not say which subtype they used.

Payne and Banatvala (1973) published a second report stating the exact environmental conditions they used in response to those people who could not reproduce their results. Seeds were sprinkled on soil in 15 cm. pots and

allowed to germinate for 2 to 4 weeks at 22°C and 70% RH. They were transplanted when they were approximately 1 cm. in height. Thirty-five plants of the same size were put in each tray. The first true leaves were to be 1.5 cm. long at time of inoculation and the plants' susceptibility to infection was enhanced by a 24 hour growth phase in the absence of light immediately before inoculation. They also reported that strains of N. sylvestris differ greatly.

Howard and McCarthy (1974) used three types of tobacco plants in attempting to reproduce the experiment. They used N. sylvestris, N. tabaccum, variety xanthi and N. tabaccum variety white burley. They failed to obtain positive results. They did report that the inoculation procedure itself could irreversibly damage young plants and this damage might be mistaken for necrosis. They did not get any effect using purified antigen at high concentrations, so it was postulated by them that it was not toxic to plants.

Dhaliwal et al. (1975) used the tobacco plant, N. sylvestris and the cowpea, Vigna sinensis to study the effect of HBAg. They did not get lesions on either type of plant but they did get some inconsistent data showing slight stunting of some of the tobacco plants.

In india, Kelkar and his associates (1975) repeated Banatvala and Payne's experiment using two types of tobacco plants - Nicotiana tabaccum variety xanthi and N. tabaccum

variety white bush. They found that the variety white bush was very resistant to the $HBAg^+$ sera with no signs of lesions being present after inoculation. The variety xanthi, however, showed lesions in some of the inoculated plants. Attempts for serial passage were unsuccessful. Extracts of the affected plants were positive for HBAg when tested by counterimmunoelectrophoresis but negative by agar gel diffusion.

CHAPTER III

MATERIALS AND METHODS

The following procedure was used to study morphologically and histologically the effect of Hepatitis B antigen treatment on Nicotiana sylvestris and Nicotiana tabaccum variety xanthi.

SEED GERMINATION

Seeds of Nicotiana sylvestris and Nicotiana tabaccum variety xanthi were spread evenly onto separate trays (Size: $8\frac{1}{2}$ x 12 x 3 inches). Each tray contained a one-half inch bottom layer of vermiculite and approximately two inches of a one-to-one mixture of potting soil and peat moss. The seeded trays were kept in a regulated growth chamber at 22°C and 70% relative humidity (RH). The seeds were allowed to germinate for a period of one to two months.

TRANSPLANTING

The seedlings (Size: 1.0 to 1.5 cm) were transferred to individual pots (Size: 3 x 3 x 4 inches). The plants were allowed to grow in the growth chamber at 22°C and 70% RH, until they attained the proper size for experimentation.

The preliminary study using Nicotiana sylvestris

used three sizes of plants. The sizes were determined by the length and width of the first leaf. The sizes chosen were 1.5 x 2.0 cm. (approximately 3 months old): 3.0 x 5.0 cm. (approximately 5 months old): and 5.0 x 8.0 cm. (approximately 7 months old).

In all subsequent experiments, the first leaf size chosen was 1.5 x 2.0 cm.

PREPARATION OF THE PLANTS FOR EXPERIMENTATION

Twelve plants of each size were used per experiment (except for the pilot study using N. tabaccum variety xanthi where twelve plants could not be obtained so four were used). The plants were subjected to a 24 hour growth phase in the absence of light, to increase susceptibility to viral infection (Payne and Banatvala, 1973). Plants were dusted with 300 mesh carborundum. To get an even distribution of carborundum on each and every leaf, plants were placed in a wooden box (2 x 3 x 1½ ft.) especially made for this purpose. The carborundum was applied with a sprayer-type air pressure gun through a small hole in the box and the carborundum was allowed to settle down on the leaves. Four plants of each size group were used for each treatment. Hepatitis B Antigen (HBAg), (purified antigen was obtained from St. Thomas Hospital, University of London, and was diluted 1:5120) hepatitis B antigen negative sera and water were used as treatments. Inoculation was achieved by placing

0.1ml. of the treatment on each leaf and rubbing the leaf lightly with a double-gloved finger. Inoculated plants were again placed in the growth chamber for post inoculation observations.

TYPE OF OBSERVATIONS TAKEN ON EXPERIMENTAL PLANTS

The gross morphology of each plant was observed and recorded at zero time and every three days thereafter for thirty-six days. The observations made were: height of the plant from the soil level to the growing plummule, size of the first leaf, size of the internodes, number of leaves, checking the leaves with a magnifying glass for the presence of lesions, and the general health of the plant (such as coloration, leaf wilting, etc.).

PREPARATION OF THE TISSUE FOR HISTOLOGICAL STUDY

Samples of the leaf tissue (1.0 x 1.0 cm.) were collected at zero time and every seventy-two hours thereafter for thirty-six days (in the pilot study, samples were taken from each size plant daily for twenty-eight days) for histological studies.

Leaf samples were washed with distilled water to remove excess dirt and carborundum and then were placed into a small vial (20ml. capacity) containing fixative. The fixative used was a mixture of formalin (5ml. commercial), acetic acid (5ml. glacial), and 50% ethyl alcohol (90ml.).

The tissue was kept in the fixative a minimum of four hours.

The tissue was removed from the fixative and was briefly washed in several changes of 50% alcohol.

DEHYDRATION OF THE TISSUE

The tertiary butyl alcohol (TBA) series was used to dehydrate the tissue. The tissues were placed into each member of the following series for two to four hours:

1. 50ml. H_2O, 40 ml. 95% Alcohol, 10 ml. TBA
2. 30ml. H_2O, 50 ml. 95% Alcohol, 20 ml. TBA
3. 15ml. H_2O, 50 ml. 95% Alcohol, 35 ml. TBA
4. 0ml. H_2O, 45 ml. 95% Alcohol, 55 ml. TBA

The tissues were then placed in three changes of 100% TBA for eight hours each. (Note: the TBA had to be kept in a warm place as its freezing point is near room temperature). At this point, the tissue was considered to be completely dehydrated.

INFILTRATION AND EMBEDDING

The tissue was removed from the TBA and was placed into a small beaker (40ml. capacity) containing melted paraffin. The beaker was then placed in the vacuum oven (Tissue-Tek Paraffin Infiltrator, Scientific Products). The oven was set at 60°C and the vacuum pump was run until the pressure reached 15 pounds. After one hour, the original paraffin was replaced by fresh paraffin, and the pressure

was held at 15 pounds at 60°C for another hour. It was believed that all air spaces had been filled with paraffin at this time.

A clean dry metal base mold was sprayed with mold release (Tissue-Tek Releasing Spray, Scientific Products) and the mold was air dried. Mold release facilitates removing the hard paraffin block from the mold. A white plastic embedding ring (LabTek Products) was placed on top of the base mold. It was then one-half filled with melted paraffin. The tissue was transferred into the paraffin with a clean forceps and melted paraffin was added to completely fill the mold. The tissue was oriented with a warm inoculating needle. The mold was then placed on the cooling tray of the Tissue-Tek II Paraffin Embedder (Scientific Products) until the paraffin hardened. The block was then removed from the mold.

The paraffin block was trimmed with a single-edged razor blade, removing as much excess paraffin as possible. In trimming, it was necessary to retain a rectangular shape in order to form even ribbons.

SECTIONING THE TISSUE

The trimmed block was then secured in the standard rotary microtome (American Optical). A newly sharpened knife was secured on the microtome and the safety latch was put in operation while adjusting the block. The block

was aligned so that the edge of the block being cut first is parallel to the knife and so that the tissue was being cut to give ninety-degree cross-sections of the leaf. The microtome was set to cut at a thickness of 5um. for the orcein stain and 10um for the general cytological investigation. The safety latch was removed and sections were cut until ribbons formed. As the ribbon formed, it was held away from the knife with a needle. The ribbons were placed on clean, frosted on one end microscope slides which were coated with a thin layer of Mayer's fixative (50% egg albumin, 50% glycerine and a preservative). It was sometimes useful to add a few drops of 4% formalin to float the ribbons to the exact part of the slide desired. The slides were then placed on a slide warming tray (Labline, Inc.) which allowed the tissue sections to spread out. The slides were allowed to cool and were air dried overnight.

STAINING THE TISSUE

The safranin-fast green staining procedure (Jensen, 1962) was used to determine if cytological changes occurred due to the hepatitis viral infection. (See Appendix A for exact procedure.) In this method, the nucleoli, chromosomes, cuticle and lignified cell walls stain red and the remaining structures stain green.

A modified orcein staining procedure (Shikata et al. 1974) was used on all remaining slides to ascertain whether

or not the hepatitis B antigen was present within the cells. (See Appendix B for exact procedure). The slides were examined for aggregates of purplish-brown colored Australia antigen. Controls were also processed for comparison purposes.

HISTOLOGICAL AND HISTOCHEMICAL OBSERVATIONS

Slides were examined with the Zeiss Binocular Microscope (Model No. 430 3464) using different magnifications. Antigen treated tissues and controls were compared to see if there were any cytological differences. Tissues were also checked for the presence of Australia antigen.

SERIAL TRANSMISSIONS

Two leaves from each type of plant showing atypical necrosis were ground separately using a cold mortar and pestle, using a small amount of carborundum (to break the cells). Grinding was continued until a sap was obtained. Healthy plants of both types were subjected to a ten hour dark period, dusted with carborundum and inoculated separately with HBAg positive sap of the same species of plant. The plants were checked daily to determine if necrosis was transmitted to the healthy plants.

PHOTOGRAPHY

Pictures of the plants were taken with a Nikkormat

Camera (Nikon) using 35mm Kodachrome 64 (Kodak) color slide film.

Photomicrographs of the stained slides were taken with the Zeiss microscope attached to an Olympus-Tokyo (Brinkman Instruments) camera attachment. The film used was High Speed Ektachrome for color slides (Kodak).

Exposed films were processed through Kodak processing laboratories.

CHAPTER IV

RESULTS

No true localized lesions occurred on either the Nicotiana sylvestris nor the Nicotiana tabaccum variety xanthi. Table I summarizes the morphological results of the first set of experiments using different age groups of Nicotiana sylvestris. The seven month old plants seemed to be resistant to the HBAg. They showed no signs of infection morphologically or histologically. Shikata's orcein stain for hepatitis antigen was inconclusive for this age group. The five month old plants also seemed resistant to the HBAg. The plant showing necrosis appeared to have been damaged in the inoculation procedure. No changes were noted histologically in this age group. The three month old plants seemed to be most susceptible to infection. The plants which died, did so by the sixth day after inoculation. The plant which did not die showed brown, dried-out areas on more than half of the leaf on every inoculated leaf, but was asymptomatic on new leaves. Slight stunting was shown in the three and seven month plants thirty-five days post inoculation. At the end of the observation period, the plants were transferred to the greenhouse where observations continued for approximately two months.

TABLE 1: EFFECT OF PURIFIED HBAg ON THE RATE OF GROWTH, SIZE OF LEAVES AND DEVELOPMENT OF NECROSIS IN THREE DIFFERENT AGE GROUPS OF *Nicotiana sylvestris*

AGE GROUP	TREATMENT	NUMBER OF PLANTS TESTED	AVERAGE HEIGHT (cm) AT ZERO TIME	STANDARD DEVIATION FOR PLANT HEIGHT AT ZERO TIME	AVERAGE HEIGHT (cm) 35 DAYS POST INOCULATION	STANDARD DEVIATION FOR PLANTS HEIGHT, 35 DAYS POST INOCULATION	AVERAGE NUMBER OF LEAVES 35 DAYS POST INOCULATION	AVERAGE SIZE OF LARGEST LEAF, 35 DAYS POST INOCULATION	NUMBER OF PLANTS SHOWING NECROSIS
3 Mos.	Water (Control)	4	1.5	0.06	2.8	0.34	5	3x5 cm	0
	HBAg- sera	4	1.6	0.13	2.9	0.63	5	3x4 cm	0 and 1 died
	HBAg+ (Pure)	4	1.4	0.22	1.7	0.00	4	2x4 cm	1 atypical 3 died
5 Mos.	Water (Control)	4	3.0	0.26	5.2	0.29	9	4x9 cm	0
	HBAg- sera	4	3.2	0.16	5.3	0.47	10	4x10 cm	0
	HBAg+ (Pure)	4	3.3	0.20	5.2	0.31	9	3x8 cm	1 atypical
7 Mos.	Water (Control)	4	5.0	0.17	7.3	0.84	15	8x15 cm	0
	HBAg- sera	4	4.8	0.40	6.8	0.46	13	7x15 cm	0
	HBAg+ (Pure)	4	5.1	0.37	6.2	0.63	17	8x14 cm	0

The preliminary study on Nicotiana tabaccum variety xanthi was run for forty-eight days. The antigen positive plants showed necrotic symptoms within six days after inoculation. Results are summarized in Table 2. This species showed severe stunting in the HBAg treated plants.

Table 3 is a summary of the two experiments performed on Nicotiana sylvestris using plants which were approximately three months old. In both experiments, necrosis was seen in all the plants by day three. Figure 1 shows control plants on the third day after inoculation. The antigen treated plants are shown in Figure 2. The leaves shriveled and were discolored. They were examined under a dissecting microscope to show the details of the tissue damage. The plants varied in the amount of necrosis caused by the antigen. By day six (Figure 3), some of the antigen treated plants had begun to recover. No necrosis was observed on new leaves. Figure 4 shows control plants six days after treatment. Figure 5 shows no necrotic symptoms on the plants of the second experiment treated with HBAg negative sera while the HBAg positive treated plants (Figure 6) show symptoms on each plant, but all the leaves on each plant were not damaged.

Figure 7 shows an antigen treated portion of the leaf (magnified 25X) of the plant shown in Figure 8. The leaf which died as a result of HBAg positive treatment (close-up, magnified 25X) was examined and is shown in Figure 9.

TABLE 2: PRELIMINARY STUDY OF THE EFFECT OF PURIFIED HBAg ON THE RATE OF GROWTH AND DEVELOPMENT OF NECROSIS IN Nicotiana tabaccum variety xanthi

TREATMENT	NUMBER OF PLANTS TESTED	AVERAGE HEIGHT (cm) AT ZERO TIME	STANDARD DEVIATION FOR PLANT HEIGHT AT ZERO TIME	AVERAGE HEIGHT (cm) 48 DAYS POST INOCULATION	STANDARD DEVIATION FOR PLANT HEIGHT, 48 DAYS POST INOCULATION	AVERAGE NUMBER OF LEAVES 48 DAYS POST INOCULATION	AVERAGE SIZE OF LARGEST LEAF, 48 DAYS POST INOCULATION	PLANT'S GENERAL HEALTH, 48 DAYS POST INOCULATION
Water (Control)	2	1.4	0.43	23.3 cm	0.68	12	9x19 cm	Good condition; thick, firm stem; green; no wilting
HBAg+ (Pure)	2	1.5	0.57	3.8 cm	Not Applicable	6	3x5 cm	One plant was severely stunted with a yellowish leaf discoloration; one died six days post inoculation

TABLE 3: EFFECT OF PURIFIED HBAg ON THE RATE OF GROWTH, SIZE OF LEAVES AND DEVELOPMENT OF NECROSIS IN *Nicotiana sylvestris*

Exp. No.	Treatment	Number Plants Tested	Average Height (cm) at Zero Time	Standard Deviation For Plant Height at Zero Time Inoculation Period	Average Height (cm) 35 Days After Inoculation	Standard Deviation For Plant Height at the End of Inoculation Period	Average Number Leaves 35 Days After Inoculation	Average Size Largest Leaf 35 Days After Inoculation	Number of Plants Showing Necrosis (Atypical)
1	Water (Control)	4	1.2	0.22	2.8	0.37	9	6x13 cm	0
	HBAg- sera	4	1.3	0.40	2.7	0.46	10	4x11 cm	0
	HBAg+ (Pure)	4	1.2	0.18	1.9	0.32	9	4x9 cm	4
2	Water (Control)	4	0.98	0.04	2.8	0.21	10	4x9 cm	0
	HBAg- sera	4	1.08	0.08	2.7	0.19	10	4x10 cm	0
	HBAg+ (Pure)	4	0.95	0.30	2.2	0.54	9	3x8 cm	4

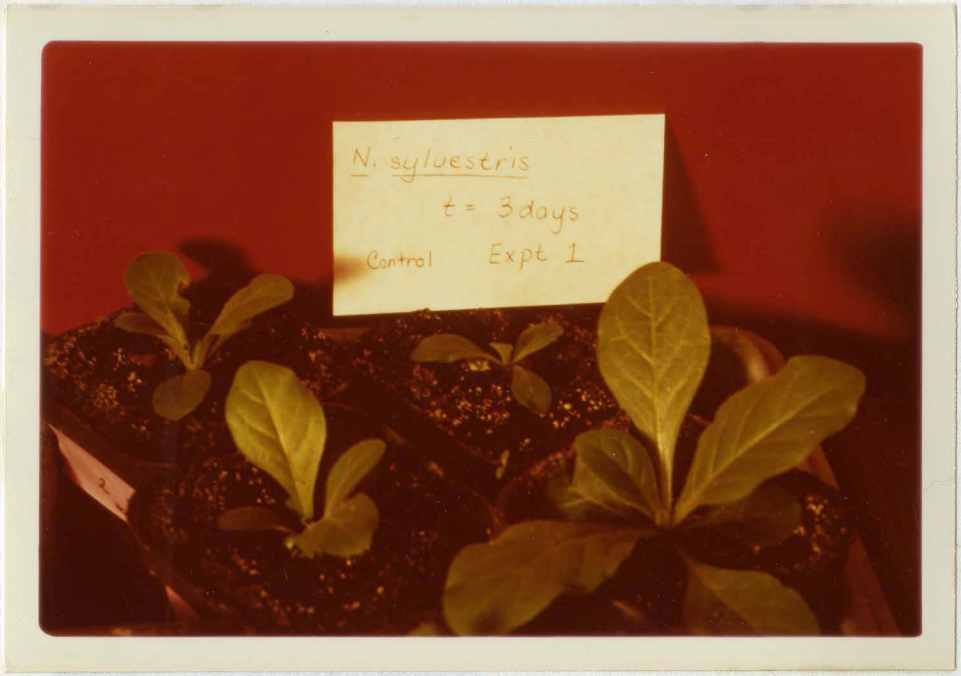

Figure 1: Nicotiana sylvestris three days after inoculating with water showing no necrosis.

Figure 2: Nicotiana sylvestris three days after inoculating with pure hepatitis B antigen showing necrosis.

Figure 3: Nicotiana sylvestris six days after inoculating with pure hepatitis B antigen showing signs of recovery from necrosis.

Figure 4: Nicotiana sylvestris six days after inoculating with water showing no necrosis.

Figure 5: Nicotiana sylvestris six days after inoculating with hepatitis B antigen negative sera showing no necrosis.

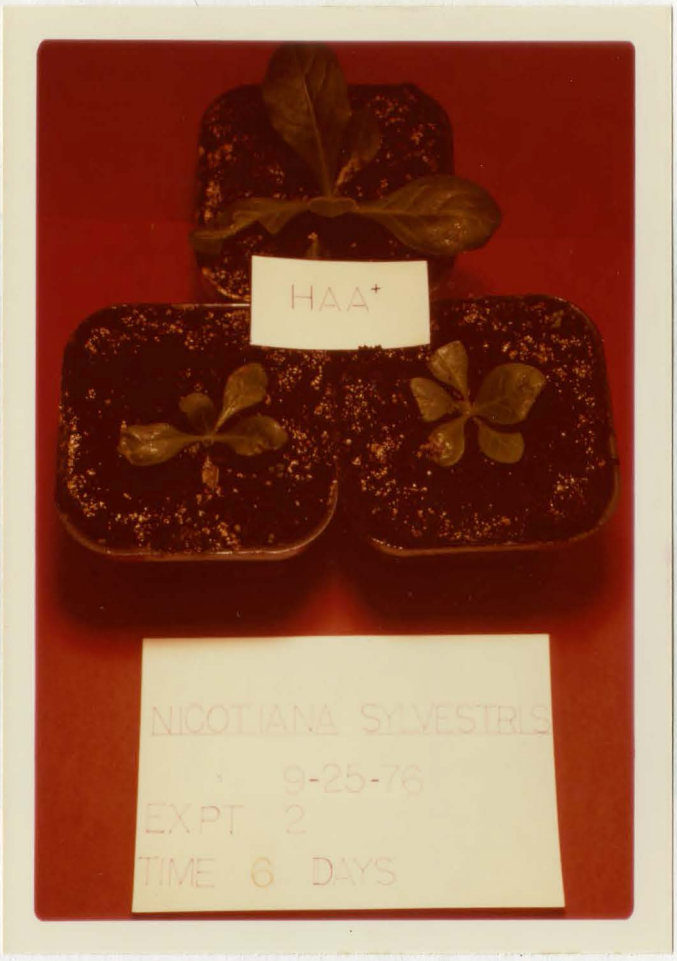

Figure 6: Nicotiana sylvestris six days after inoculating with pure hepatitis B antigen showing necrosis.

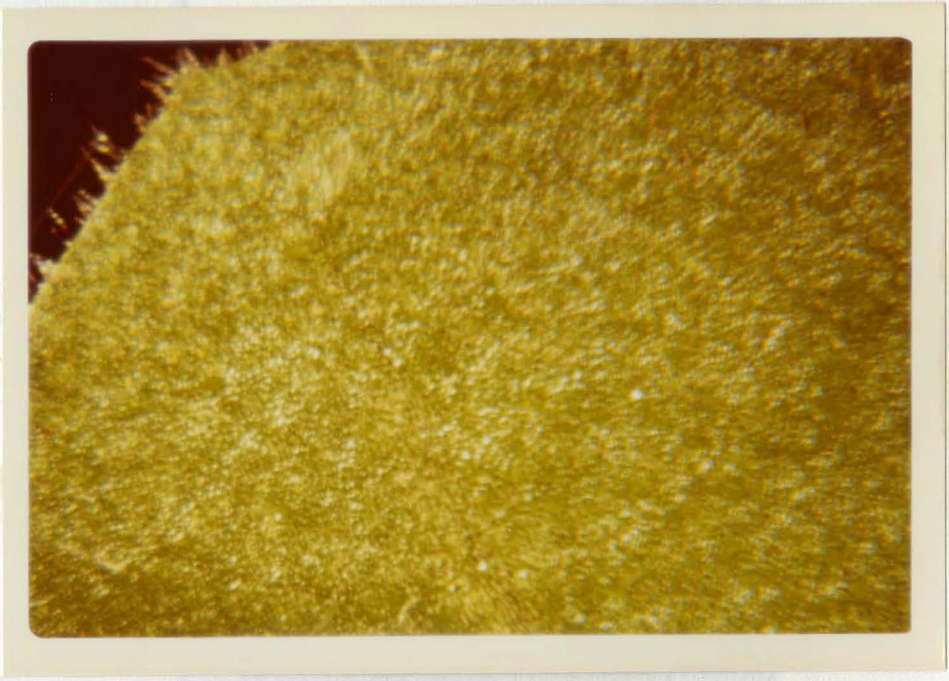

Figure 7: *Nicotiana sylvestris* treated with pure hepatitis B antigen showing portion of necrotic leaf (magnified 25X).

Figure 8: Nicotiana sylvestris six days after inoculating with pure hepatitis B antigen showing necrosis.

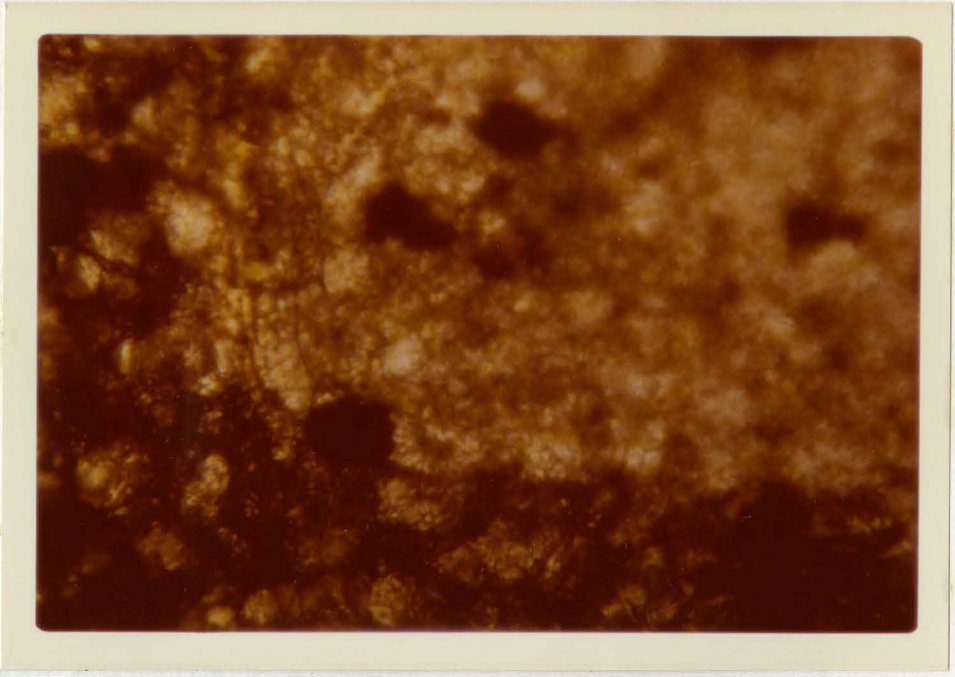

Figure 9: Portion of the necrotic leaf of Nicotiana sylvestris six days after inoculation with pure hepatitis B antigen (magnified 25X).

Figures 10 and 11 show that by day 36 the antigen treated plants were quite healthy. Histological investigation showed no change in leaves which were asymptomatic and leaves which had turned brown did not section well; cell structures were not preserved. They did not stain positively for the presence of antigen aggregates.

Necrosis was seen in two experiments with Nicotiana tabaccum variety xanthi. The results of these experiments are summarized in Table 4.

Control plants showed no evidence of discoloration or wilting (Figure 12), while each antigen positive treated plant showed some evidence of necrosis (Figure 13), by the sixth day after inoculation.

Figure 14 shows the plant which died as a result of the HBAg positive treatment.

Figures 15 and 16 (magnified 25X) show areas of infected leaves. Stunting was noticed at day 36: but the plants had recovered showing no signs of necrosis (Figure 17). Observing the plants three weeks later, the antigen positive plants were the same height as control plants. Histological studies were again inconclusive.

Attempts to transfer the necrosis causing agent from the HBAg treated plants to the healthy plants of N. sylvestris and N. tabaccum variety xanthi were made. Experimental data showed that the agent appeared to be transmittable from plant to plant.

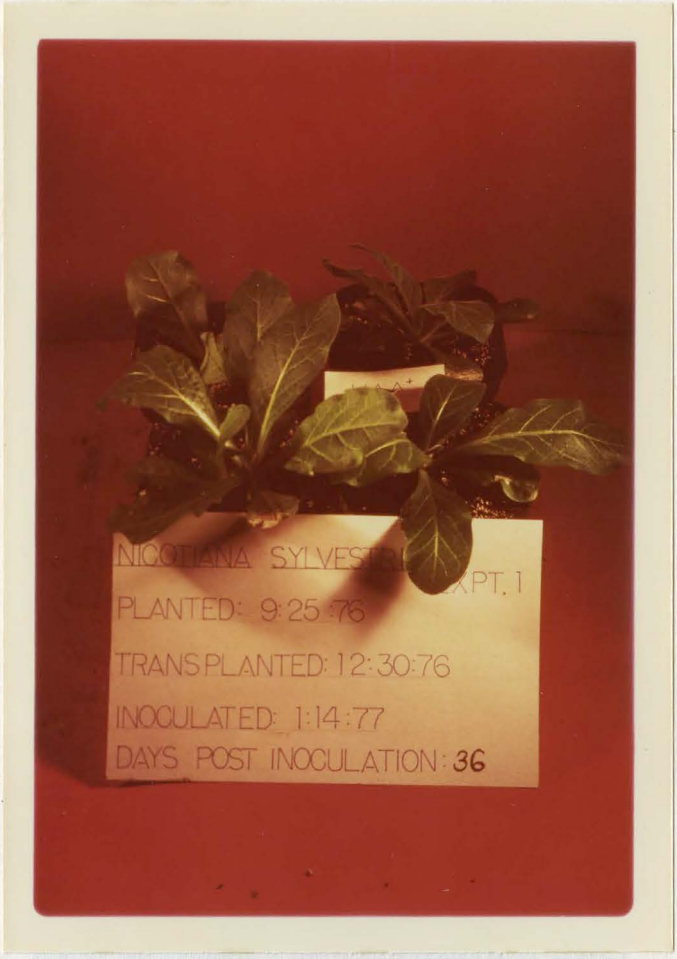

Figure 10: Nicotiana sylvestris thirty-six days after inoculating with pure hepatitis B antigen showing no necrosis.

Figure 11: Nicotiana sylvestris thirty-six days after inoculating with pure hepatitis B antigen showing no necrosis.

TABLE 4: EFFECT OF PURIFIED HBAg ON THE RATE OF GROWTH, SIZE OF LEAVES AND DEVELOPMENT OF NECROSIS IN Nicotiana tabaccum variety xanthi

Exp. No.	Treatment	Number Plants Tested	Average Height (cm) at Zero Time	Standard Deviation For Plant Height at Zero Time Inoculation Period	Average Height (cm) 35 Days After Inoculation	Standard Deviation For Plant Height at the End of Inoculation Period	Average Number Leaves 35 Days After Inoculation	Average Size Largest Leaf 35 Days After Inoculation	Number of Plants Showing Necrosis (Atypical)
1	Water (Control)	4	1.6	0.31	0.1	0.78	10	5x6 cm	0
	HBAg-sera	4	1.3	0.21	6.9	1.3	9	4x5 cm	0
	HBAg+	4	1.4	0.20	4.8	1.2	7	3x5 cm	2 and 2 died
2	Water (Control)	4	1.4	0.12	8.8	2.8	8	5x7 cm	0
	HBAg-sera	4	1.5	0.34	8.4	1.1	8	5x6 cm	0
	HBAg+	4	1.3	0.22	3.6	1.4	5	3x4 cm	4

Figure 12: Nicotiana tabaccum variety xanthi six days after inoculating with water showing no necrosis.

Figure 13: Nicotiana tabaccum variety xanthi six days after inoculating with pure hepatitis B antigen showing necrosis.

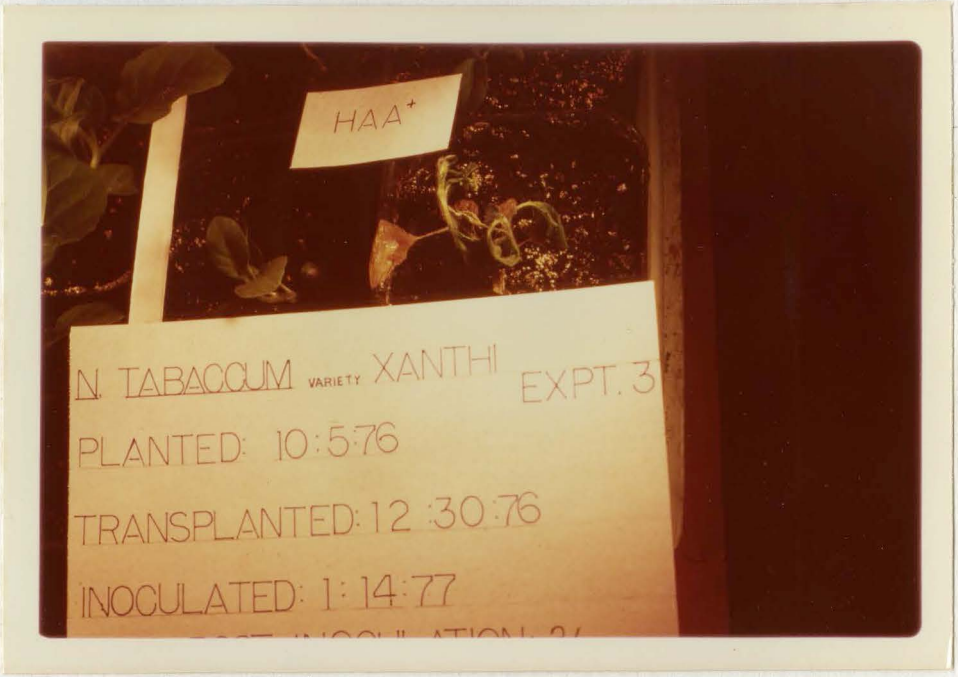

Figure 14: Nicotiana tabaccum variety xanthi showing necrosis as a result of pure hepatitis B antigen treatment.

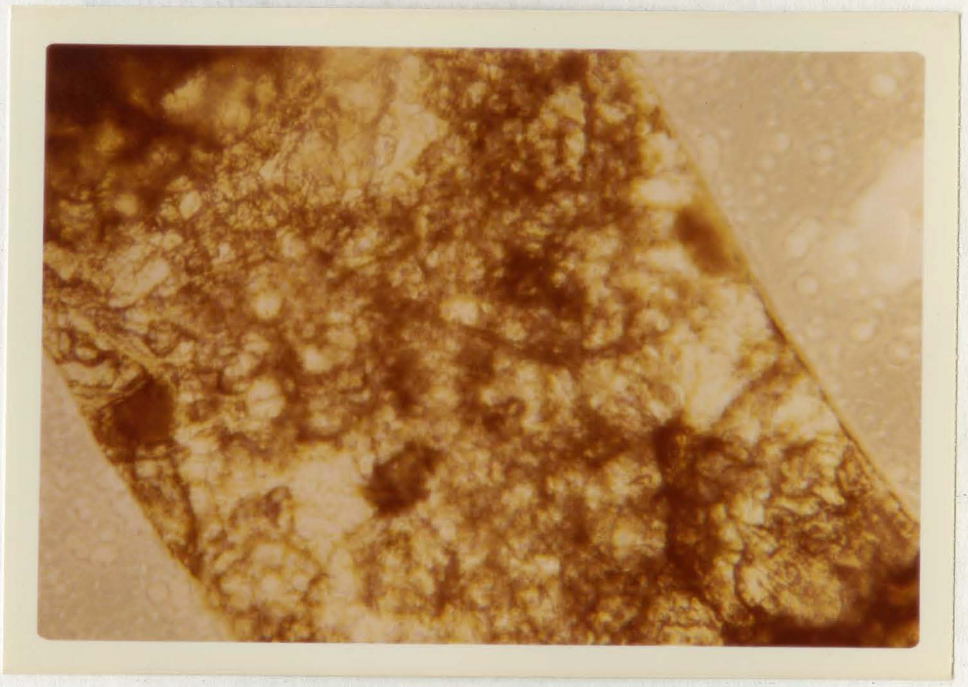

Figure 15: Portion of pure hepatitis B antigen treated leaf of Nicotiana tabaccum variety xanthi six days after inoculation showing necrosis (magnified 25X).

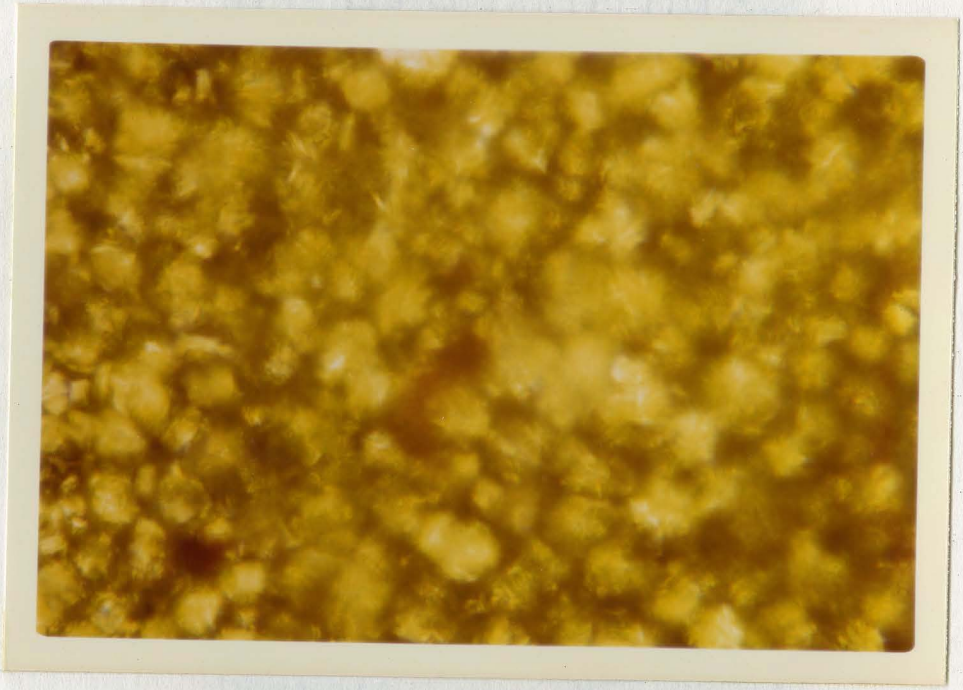

Figure 16: Portion of necrotic Nicotiana tabaccum variety xanthi leaf treated with pure hepatitis B antigen (magnified 25X).

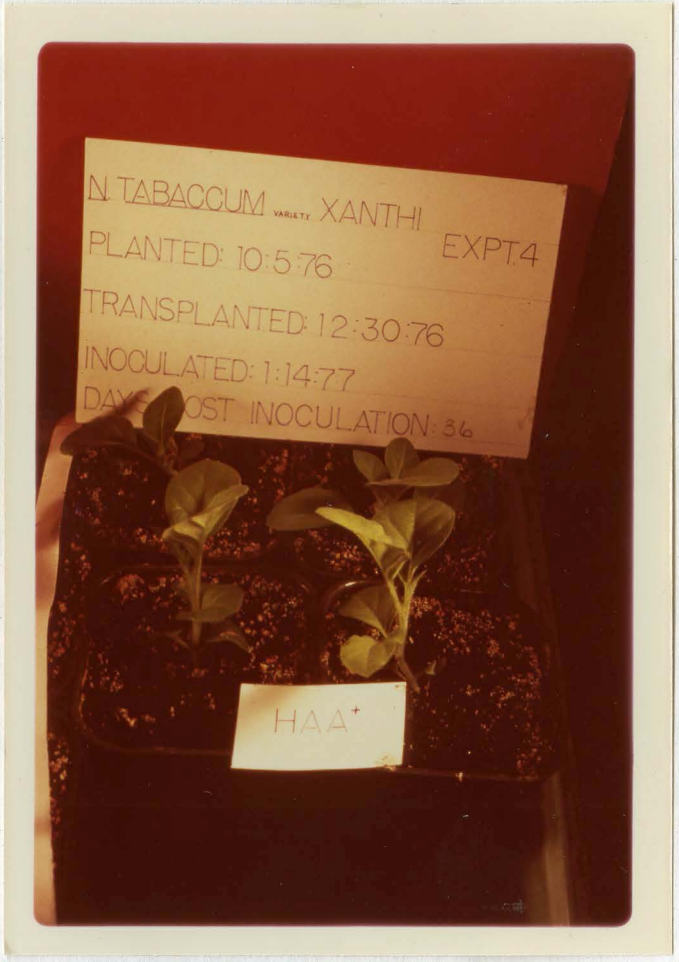

Figure 17: Nicotiana tabaccum variety xanthi thirty-six days after inoculating with pure hepatitis B antigen showing no necrosis.

CHAPTER V

DISCUSSION

If a virus is placed upon an intact leaf, infection cannot occur (Matthews, 1970). A wound or surface damage is necessary for viral entry and infection. Mechanical inoculation of the leaf surface which has been dusted with an abrasive like carborundum, damages the epidermal hairs and provides a site for viral entry. It seems that any of the epidermal cells are capable of being infected by this type of inoculation. (Matthews, 1970).

Brants (1965) postulates that viral particles may enter the leaf through the plasmodesmata (guard cells) when only the cuticle is damaged. Having used an abrasive to wound the leaf surface, it may be assumed that the hepatitis B antigen was provided a way of entry into the leaf.

If one wishes to establish the viral nature of a plant disease transmission experiments are usually performed. The two types of tobacco plants were first tested for susceptibility to the hepatitis B antigen. Three different sizes of Nicotiana sylvestris were inoculated to determine if a particular size (age) group would be more susceptible than another. It was found that five and seven month plants appeared to be quite resistant to viral infection. It must be kept in mind that this lack of effect

is not necessarily due to the specificity of the virus, but it could be due to other factors such as the presence of certain inhibitors which change with the age of the plants (Matthews, 1970).

The preliminary study performed on Nicotiana tabaccum variety xanthi was to determine if the size of plant suggested by Kelkar et al. (1975) would be susceptible to viral infection under our laboratory conditions.

There are several different ways that diseases may be expressed in plants, such as macroscopic symptoms including localized and systematic effects, and localized lesions or death of cells near the site of viral entry. Systemic viral invasion most often causes stunting of growth (Matthews, 1970). The degree of stunting may be correlated with the severity of the infection. Stunting may equally affect all parts of the plants or only some areas. Mosaic effects (patterns of two or more colors) are also common. Necrosis is the death of a group of cells, organs or the whole plant. In addition to the above abnormalities it is possible to have other types of abnormal growth as well.

Both species of plants appeared to be susceptible to localized invasion by the hepatitis B antigen. A typical localized lesion did not appear in our experimental plants, but yellow to brown patches were noticed on leaves which were inoculated with HBAg. These areas of the leaf were

composed of groups of dead and dying cells so it was described as a form of necrosis. If injury was caused by inoculation, one would have expected both water and sera controls to show these necrotic patches. This was not observed in our experiments. No necrotic patches were noticed on new leaves emerging after inoculation. This implied that the viral invasion did not show typical systemic symptoms like that of tobacco mosaic virus. Nicotiana sylvestris plants showing necrotic symptoms were slightly smaller than control plants, perhaps due to a reduction in the photosynthetic mechansim. Recovery of these plants from viral infection was observed by thirty-six days after inoculation. The Nicotiana tabaccum variety xanthi was more severely stunted by the viral invasion, but the plants seemed to recover sooner than the Nicotiana sylvestris. By twenty-five days after inoculation, they were quite healthy. It was also noted that within three weeks after the completion of the experiment, the infected plants had caught up in size to the control plants. Thus, it is possible that the HBAg somehow, due to unknown reasons, might have lost its activity.

The inability to confirm the presence of antigen histologically may be due to several factors. Shikata's orcein stain for the presence of Australia antigen may have needed longer time for adequate staining. Also, although

plant and animal cells are very similar (Krogmann, 1973), they are not identical and some staining techniques may need to be altered. It is also possible that the antigen replicating in the plant may have altered its original form. Whenever HBAg replication has been reported, strain analysis has shown wide variation. In order to determine the changes present in the original HBAg particles, a large amount must be present to be detected in the tissue, with the help of the light microscope. Perhaps a more sensitive staining procedure like Huang's immunofluorescent method (1975b) could be employed to confirm the presence of antigen. The lack of general cytological changes may be due to the wrong choice of stains. In man, cytological and pathological changes due to HBAg infection can be evidenced in liver tissue but not in certain other types of tissue, so perhaps the leaf tissue was not the place to look for such changes. Another factor to consider is that necrotic symptoms were present three days after inoculation and samples were taken every 72 hours. Perhaps the tissues were already dead and the changes in the tissue could not be detected. Such changes could only be detected before cell death. A future study might include collecting tissue samples every hour after inoculation while the cells are still in viable condition so that the changes leading to cellular death might be observed.

The infection of the plant tissue with the HBAg

seems to induce the necrotic symptoms even though it could not be shown histologically. The necrosis causing agent was transmissable from one set of plants to another. There are other factors which can cause virus-like symptoms. These include biological, chemical and physical agents. Yellowing of leaves and stunting may be caused by mycoplasma like organisms, however, these cannot be mechanically transmitted (Matthews, 1970). Systematic virus-like symptoms may be induced by arthropod toxins (Bos and Grancini, 1968). Other factors affecting the health of the plants and the presence of virus-like symptoms are genetic abnormalities, nutritional deficiencies, incorrect temperatures for growth, hormonal damages, sudden environmental changes and drug effects (Matthews, 1970).

Careful handling of the plants was used to avoid the effect of outside factors on the experimental plants. The plants of each experiment were subjected to the same environmental conditions and it seems that when the right size plants are used, they are susceptible to infection with HBAg.

SUMMARY

When three different age groups of Nicotiana sylvestris were inoculated with purified HBAg, it was found that the three month old plants were most susceptible to infection as evidenced by necrosis. Five and seven month plants showed no sign of infection. Nicotiana tabaccum variety xanthi were susceptible to HBAg infection under the environmental conditions used in the laboratory.

Four experiments were performed (two on each species of plant) and necrosis was observed on all the plants treated with purified HBAg, within the first six days after inoculation. No necrosis was observed on water control plants nor was necrosis observed on plants treated with HBAg negative sera. Slight stunting of growth was observed in Nicotiana sylvestris. Recovery from infection was observed 36 days after inoculation. Stunting was more severe in HBAg treated plants of Nicotiana tabaccum variety xanthi, and recovery from infection occurred sooner (by day 25). Three weeks after the completion of the experiment, the HBAg treated plants had achieved approximately the same height as control plants. Presence of viral infection could not be confirmed histologically.

The necrosis causing agent appeared to be transmittable (via extracts of plant sap) from plants showing necrosis to healthy plants of the same species.

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APPENDIX A

Safranin-Fast Green staining procedure used in staining Nicotiana tabaccum variety xanthi and Nicotiana sylvestris leaf tissue inoculated with pure HBAg, HBAg negative sera and water.

1. Deparaffinize tissue by placing slides in each of the following solutions for 5 minutes each:
   a. Abolute xylene.
   b. 1 part absolute xylene: 1 part absolute alcohol.

2. Partially hydrate tissue by placing slides in following solutions for 5 minutes each:
   a. Absolute alcohol.
   b. 95% alcohol.
   c. 75% alcohol.
   d. 50% alcohol.

3. Stain slides in Safranin for 4 hours.
   Safranin solution was made by preparing a stock solution of 3% safranin in 95% alcohol.

4. The slides were washed off with water and were placed into each of the following solutions for 15 seconds:
   a. acidified 70% Alcohol (3%HCl to destain).
   b. 95% alcohol (to dehydrate).
   c. absolute alcohol (completion of dehydration).

5. Sections were counterstained with fast-green for 10 seconds.
   Fast Green Stain is: 0.5% fast green in a solution which is 50% clove oil and 50% alcohol.

6. The sections were differentiated by two five minute changes in a solution which was 50% clove oil, 25% absolute alcohol and 25% xylene.

7. The remaining paraffin was removed by three 15 minute changes of xylene.

8. A coverslip was mounted using Pro-Mount (Scientific Products).

APPENDIX B

Modified orçein staining procedure * used in staining HBAg aggregates in mesophyll cells of Nicotiana tabaccum variety xanthi and Nicotiana sylvestris leaf tissues.

1. The slides were deparaffinized by two ten-minute changes of xylene.

2. The tissue was hydrated to water by placing the slides in each of the following for 5 minutes:
   a. Absolute alcohol.
   b. 95% alcohol.
   c. 70% alcohol.
   d. 50% alcohol.
   e. 30% alcohol.
   f. Water.

3. Due to the presence of a cell wall, the tissue was oxidized to facilitate staining. This procedure also decolorizes the cytoplasm.
   a. Slides were placed in 0.3% Potassium Permanganate (Sigma Chemicals) solution (with 12 Normal Sulfuric Acid) for 5 minutes.
   b. The slides were then completely decolorized in 1.5% oxalic acid (Sigma Chemicals).

4. The slides were stained for four hours in acidified orcein (1g. orcein in 100ml. 70% alcohol pH adjusted to 2 with HCl). Excess stain was washed off with water.

5. The slides were placed in absolute alcohol and were checked microscopically every 5 minutes for an acceptable color contrast.

6. The slides were passed through three fifteen minute changes of xylene to make sure all paraffin and excess particles on the slides were removed.

7. The slides were air dried, and then a coverslip was mounted using Pro-Texx Mounting Medium (Scientific Products).

* Shikata, T., T. Uzawa, N. Yoshiwara, T. Akatsuka, and S. Yamazaki. Staining Methods of Australia Antigen in Paraffin Section - Detection of cytoplasmic inclusion bodies. Japan J. Exp. Med. 44(1): 25-36. 1974.

APPENDIX C

Statistical analysis procedures used to determine the variability and significance of the data.

STANDARD DEVIATION

Standard deviation was used to measure the variability of the plant height within each treatment group; eg. the pure HBAg, HBAg⁻ sera and the water treatments.

1. The average (mean) height was determined for each group.

   Example: Experiment 2; Observations taken 35 days after treatment

Treatment	Plant Number	Height (cm.)
$HBAg^+$	7	1.8
$HBAg^+$	8	1.5
$HBAg^+$	9	2.8
$HBAg^+$	10	2.6
Sum of all four heights -		8.7 cm.

   Average height = sum ÷ number of plants
   = 8.7 cm./4 = 2.175 cm.

2. How much each value deviates from the mean was then determined (in absolute value).

   Example:

Plant No.	Height	Height-Mean	Deviation
7	1.8 cm.	1.8 -2.175	0.375
8	1.5 cm.	1.5- 2,175	0.675
9	2.8 cm.	2.8 -2.175	0.625
10	2.6 cm.	2.6 -2.175	0.425

3. Each deviation was squared; all the squares were added and the average square of deviation (6^2) was determined. This term is called the variance.

Plant No.	Deviation	Deviation Squared
7	0.375	0.1406
8	0.675	0.4556
9	0.625	0.3906
10	0.425	0.1806
Sum of deviations squared		1.1674

   Average = 1.1674 ÷ 4 = 0.2918

4. The standard deviation is the square root of the variance.

Variance = 0.2918
Standard deviation = $\sqrt{0.2918}$=0.540

A standard deviation of less than 1 was considered acceptable.

ANALYSIS OF VARIANCE

Analysis of variance tests were performed on the data from each experiment to ascertain if the differences observed were significant, eg. Experiment 4: Nicotiana tabaccum variety xanthi.

1. The plant heights measured 35 days after inoculation were tabulated in the following way:

X_1 = height of plant
X^2 = square of plant height
Sum = addition of values in that column

	Water Control		Negative Sera		$HBAg^+$ (pure)	
	X_1	X_1^2	X_2	X_2^2	X_3	X_3^2
	4.0	16.	8.3	68.9	2.5	6.3
	10.0	100.	11.0	121.	2.0	4.0
	10.2	104.	6.1	37.2	5.0	25.
	11	121.	8.0	64.	5.0	25.
Sum	35.2	341.	33.3	291.1	14.5	60.3

2. The number of plants observed in each treatment were determined and designated by "n" so,

$n_1 = 4$; $n_2 = 4$; $n_3 = 4$

3. The average of the plant height was obtained by dividing the sum of plant height by n.

$$\bar{X}_1 = \frac{\sum X_1}{n_1} \qquad 35.2/4 = 8.8$$

$$\bar{X}_2 = \qquad 33.3/4 = 8.3$$

$$\bar{X}_3 = \qquad 14.5/4 = 3.6$$

4. All the plant heights were added together (ΣX_{tot}) and all the squares of plant height were added together.

(ΣX^2_{tot})

$$\Sigma X_{tot} = X_1 + X_2 + X_3$$

$$+ 35.2 + 33.3 + 14.5 = 83$$

$$\Sigma X_{tot}^2 = X_1^2 + X_2^2 + X_3^2$$

$$+ 341 + 291.1 + 60.3 = 392.4$$

5. The total number of plants observed in the experiment was obtained by adding the number of plants in each treatment.

$$N = n_1 + n_2 + n_3$$

$$= 4 + 4 + 4 = 12$$

6. The total sum of squares was obtained, with the following formula;

$$\Sigma X^2_{tot} = \Sigma X^2_{tot} - (\Sigma X_{tot})^2/N$$

$$= 692.4 - \frac{(83)^2}{12}$$

$$= 692.4 - \frac{6889}{12}$$

$$= 692.4 - 574.1 = 118.3$$

7. The between group sum of squares for three groups was determined;

$$\Sigma X^2_B = \frac{(\Sigma X_1)^2}{n_1} + \frac{(\Sigma X_2)^2}{n_2} + \frac{(\Sigma X_3)^2}{n_3} - \frac{(\Sigma X_{tot})^2}{N}$$

$$= \frac{(35.2)^2}{4} + \frac{(33.3)^2}{4} + \frac{(14.5)^2}{4} - \frac{(158)^2}{12}$$

$$= 309.76 + 277.2 + 52.56 - 574.1$$

$$+ 65.42$$

8. The within-group squares was obtained for three group analysis by subtracting the between group sum of squares from the total sum.

$$\Sigma x_W^2 = 118.3 - 65.42 = 52.88$$

9. The between group variance estimate was determined by dividing the between group sum of squares by the degrees of freedom (df_B).

df_B = The number of groups - 1

= 3 - 1 = 2

$$\text{variance estimate 1} = \frac{65.42}{2} = 32.71$$

10. The within group variance estimate is determined by dividing the within group sum of squares by the degrees of freedom (df_W)

df_W - total number of observations minus the number of groups

= 12 - 3 = 9

$$\text{estimate of variance} = \frac{52.88}{9} = 5.87$$

11. The value of F is found by dividing the between group variance estimate by the within group variance estimate:

$$F = \frac{32.71}{5.87} = 5.57$$

12. This value is checked on a table of F values to determine significance. For the number of degrees of freedom in this experiment, any F valve greater than 4.26 is significant at the 0.05 level.

Therefore, the difference in height due to pure hepatitis B antigen treatment in this experiment is significant.

Reference: Runyon, R. P. and A. Haber. Fundamentals of Behavioral Statistics 3rd ed. Wesley Pub. Co. pp. 293-295. 1976.

APPROVAL SHEET

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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science in Biology.

5/5/1977
Date

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